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14. ABSTRACT The goal of this project is to characterize the role of NF- B2/p52 in the aberrant activation of AR signaling in castration-resistant prostate cancer. Our preliminary data demonstrate that NF-kB2/p52 is expressed at high levels in prostate cancer and the overexpression of NF-kB2/p52 facilitates castration resistant prostate cancer progression by activation of AR signaling and rescue of cancer cells from apoptotic death induced by androgen deprivation. Our hypothesis is that NF- B2/p52 activates the AR and protects prostate cancer cells from apoptotic cell death induced by androgen deprivation therapy, leading to the development of castration resistance prostate cancer. The specific aims are: 1. To determine the role of p52 activation in androgen responsiveness and progression of castration resistant prostate cancer. 2. To determine the effect of expression of p52 on the development and progression of prostate cancer in a transgenic mouse model. 3. To determine the mechanisms of AR activation by p52.					
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Introduction

The goal of this project is to characterize the role of NF- κ B2/p52 in the aberrant activation of AR signaling in castration-resistant prostate cancer. The growth of prostate cancer is initially dependent on androgen and can be effectively treated by androgen-deprivation therapy. However, androgen-deprivation therapy only causes a temporary regression of prostate cancer, as all tumors will eventually progress to refractory to hormonal therapy (castration resistant prostate cancer). Androgen signaling through androgen receptor (AR) plays an important role not only in maintaining the function of the prostate, but also in promoting the development of androgen-independent prostate cancer. AR signaling is often hyperactive in androgen-independent prostate cancer. Our preliminary data demonstrate that NF- κ B2/p52 is expressed at high levels in prostate cancer and that overexpression of NF- κ B2/p52 facilitates castration resistant prostate cancer progression by activation of AR signaling and rescue of cancer cells from apoptotic death induced by androgen deprivation.

Progress Report

We have made significant progress in Task 1 (i.e., Determination of whether knockdown of endogenous p52 expression in androgen insensitive C4-2 and LNCaP-IL6+ cells can block tumor growth).

Downregulation of p52 inhibits prostate cancer cell proliferation We obtained shRNAs targeting p52 from Open Biosystems. The efficacy of these shRNAs to downregulate p52 expression was verified by western blotting. C4-2 cells, which express higher levels of p52 compared to LNCaP, were transfected with plasmids encoding p52 shRNA and growth was monitored in FBS and CS-FBS conditions. Downregulation of p52 inhibited cell growth of C4-2 cells in both FBS and CS-FBS conditions (Fig. 1A). Expression of PSA was measured using ELISA in cell supernatants and was found to be lower in C4-2 cells transfected with p52 shRNA compared to vector controls (Fig. 1C). Similarly, LNCaP-IL6+ cells were transfected with p52 shRNA and cell growth was monitored in FBS and CS-FBS conditions. Downregulation of p52 inhibited growth and PSA secretion in LNCaP-IL6+ cells (Fig. 1B & C).

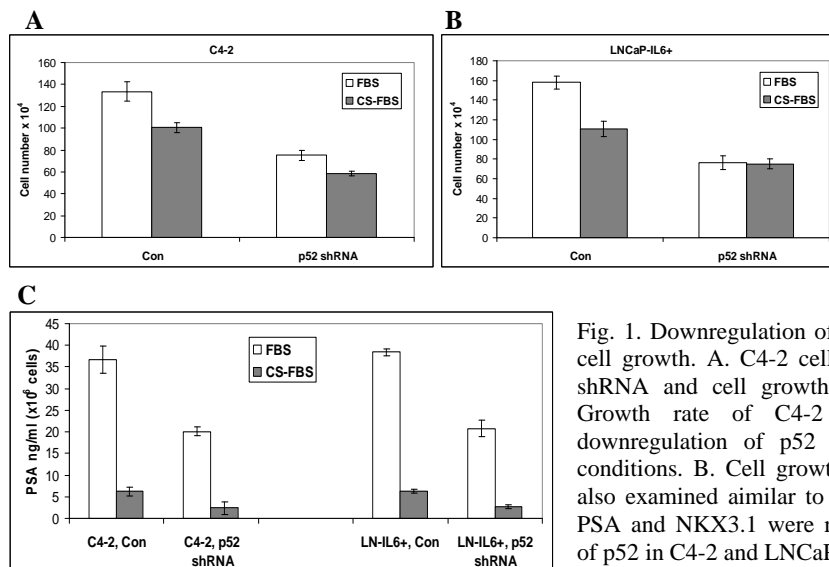


Fig. 1. Downregulation of p52 inhibits prostate cancer cell growth. A. C4-2 cells were transfected with p52 shRNA and cell growth was measured after 48 h. Growth rate of C4-2 cells was reduced after downregulation of p52 in both FBS and CS-FBS conditions. B. Cell growth of LNCaP-IL6+ cells was also examined similar to (A). C. Expression levels of PSA and NKX3.1 were reduced after downregulation of p52 in C4-2 and LNCaP-IL6+ cells.

Generation of cell lines stably expressing p52 We have also successfully generated LNCaP cell lines both constitutively and inducibly expressing p52. LNCaP cells were transfected with pCDNA3.1-HA-p52 and stable clones were generated after selection with G418. Expression of p52 was verified using anti-HA and anti-p52 antibodies. To generate LNCaP cells exhibiting Tet-inducible expression of p52, p52 ORF was cloned into the pLenti-TO/V5/DEST/Flag lentiviral vector using the ViraPower tetracycline-inducible lentiviral expression system (Invitrogen). The plasmid was verified by sequencing and was used to generate lentiviruses after packaging with the packaging plasmid mix (Invitrogen). The resultant lentiviruses were used to transduce LNCaP/TR cells expressing the Tet repressor. Stable clones were generated by selection with blasticidin/zeocin. Expression of p52 after induction with doxycycline in the stable clones was confirmed using anti-Flag and anti-p52 antibodies (Fig. 2A). Expression of androgen-induced genes like PSA and NKX3.1 was found to be induced in LNCaP cells expressing tet-inducible p52 compared to vector controls (Fig. 2B).

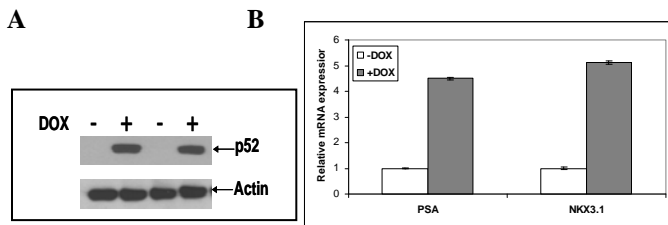


Fig. 2. A. pLenti4/TO/V5/DESt-Flag-p52-containing lentiviruses were used to infect LNCaP cells and stable clones were selected with zeocin. Expression of p52 was induced by induction with doxycycline. B. Expression of p52 was induced by doxycycline in LNCaP cells expressing tet-inducible p52 and expression levels of PSA and NKX3.1 mRNAs were analyzed by qRT-PCR. Induction of p52 expression led to an increase in expression levels of these two AR-responsive genes.

We have made significant progress in Task 3 (i.e. Determine the mechanisms of AR activation by p52).

3a. Determination of whether p52 enhances AR nuclear translocation.

To determine whether p52 affects expression of AR directly, LNCaP and LAPC-4 cells were grown in CS-FBS for 2 days and transfected with p52-expressing plasmids. Whole-cell extracts were analyzed with anti-AR antibodies. The results showed that p52 did not enhance the expression of AR (Fig. 5A). We also analyzed AR mRNA expression in LNCaP and LAPC-4 cells transfected with p52 in CS-FBS using real-time quantitative RT-PCR. We found that overexpression of p52 did not affect AR expression at the level of mRNA. The AR typically translocates from the cytoplasm to the nucleus on activation. To examine whether p52 enhances nuclear translocation of the AR protein in the presence or absence of low levels of androgen, nuclear extracts from p52-transfected LNCaP cells grown in CS-FBS were analyzed by immunoblotting with anti-AR antibodies. The level of nuclear translocation of AR under conditions of androgen deprivation was very low, which was enhanced with the expression of p52 (Fig. 5B). To further confirm these results, LNCaP cells transfected with p52 or control were processed for immunofluorescent staining with AR antibodies. AR was confined mainly to the cytoplasmic compartment in control cells in CS-FBS, whereas stronger nuclear staining of AR was observed in p52-transfected cells, although some AR was still seen in the cytoplasm (Fig. 5C, left panels). To verify that these results were not due to the T877A mutation present in the AR in LNCaP cells, we cotransfected AR-negative PC-3 cells

with Wt-AR and p52 followed by immunofluorescent staining with AR antibodies. AR expression was mostly seen in the cytoplasmic compartment in control cells, whereas it was mostly located in the nucleus in p52-transfected cells (Fig. 5C, right panels). These results suggested that p52 may facilitate the nuclear translocation of AR during androgen deprivation.

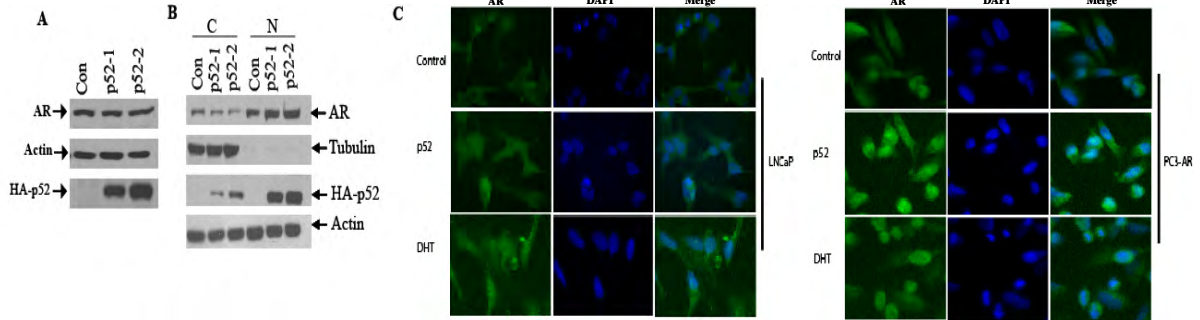


Fig. 5. p52 induces nuclear translocation of AR. Androgen-deprived LNCaP cells were transfected with HA-p52 and control vector. Whole cell (A) and cytoplasmic (C) and nuclear (N) (B) proteins were immunoblotted with AR, Actin, tubulin or HA antibodies. Actin and Tubulin were used as loading controls for whole cell and cytoplasmic extracts respectively. C. Androgen-deprived LNCaP cells were transfected with p52 plasmid (1 or 2 mg) or control vector, or treated with 1 nM DHT for 24 h (left panels). PC-3 cells were transfected with Wt-AR and HA-p52 in CS-FBS for 24 h (right panels). Cells were processed for immunofluorescent staining of AR, and nuclei were stained with DAPI.

3b. Determination of whether p52 facilitates AR binding to the androgen responsive elements (ARE) of the target genes.

NF- κ B2/p52-activated AR is preferentially recruited to the PSA distal enhancer.

The PSA promoter in the PSA-Luc construct contains two sets of AREs: AREI/II (the proximal enhancer) located at -0.1 kb and AREIII (the distal enhancer) located at -6 kb. The ARE-Luc construct contains only AREI/II (the proximal enhancer). To determine whether p52 modulates recruitment of AR to the PSA promoter, ChIP assays were performed to examine the recruitment of AR by p52 and DHT. As shown in Fig. 6A, p52-activated AR binds only to the distal enhancer (AREIII), whereas DHT-bound AR is recruited to both ARE sites. To test whether the differential recruitment of AR to AREs by p52 translates to differential AR activation, we performed luciferase assays using reporters containing the enhancer element (AREIII-Luc), the proximal AREs (AREI/II-Luc), or the full-length PSA promoter (PSA-6.0-Luc). We found that p52 induced the activity of AREIII-Luc and the full-length promoter but not AREI/II-Luc. As expected, DHT stimulates the activity of AREI/II-Luc, AREIII-Luc, and PSA-6.0-Luc (Fig. 6B). These data showed that p52-activated AR is preferentially recruited to the PSA distal enhancer.

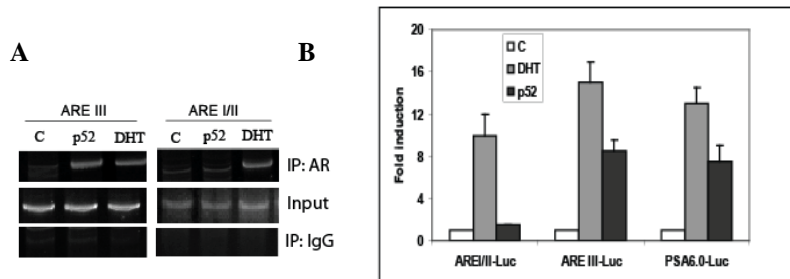


Fig. 6. A. p52 recruits AR preferentially to the distal enhancer of PSA promoter. Androgen-deprived LNCaP cells were transfected with plasmids expressing p52 and vector \pm 1 nM DHT. ChIP assays were performed using AR antibodies. B. Androgen-deprived LNCaP cells were co-transfected with p52 and pGL3-AREI/II-Luc, pGL3-AREIII-Luc or pGL3-PSA-Luc \pm 1 nM DHT for 48 h and luciferase activities were measured.

NF- κ B2/p52-induced PSA expression is dependent on AR expression.

Previous studies showed that NF- κ B-p50 complex binds directly to the distal core enhancer region in the PSA promoter and enhances PSA expression. So, we examined whether p52 binding to the AREIII element in the PSA enhancer is AR dependent or independent. Total RNAs were isolated from LNCaP cells transfected with plasmids encoding p52 and AR siRNA, and PSA mRNA expression was analyzed by quantitative RT-PCR (qRT-PCR). We found that p52 enhanced PSA mRNA expression ~30-fold in CS-FBS, which was almost completely abolished by downregulation of AR (Fig. 7A). This was also confirmed using ELISA, where p52 induced the secretion of PSA by LNCaP cells in CS-FBS, which was abolished by downregulation of AR (Fig. 7B). To confirm that the recruitment of p52 to AREIII is AR dependent, we performed ChIP assay with lysates from LNCaP cells transfected with p52 and AR siRNA in CS-FBS. The results showed that recruitment of p52 to the AREIII site was completely AR dependent (Fig. 7C, ChIP), which was consistent with the above results. Lysates from these cells were analyzed by immunoblotting with anti-AR antibodies to confirm that AR expression was downregulated >70% in AR siRNA-transfected cells (Fig. 7D, WB). These findings clearly showed that the p52-enhanced PSA expression is AR dependent.

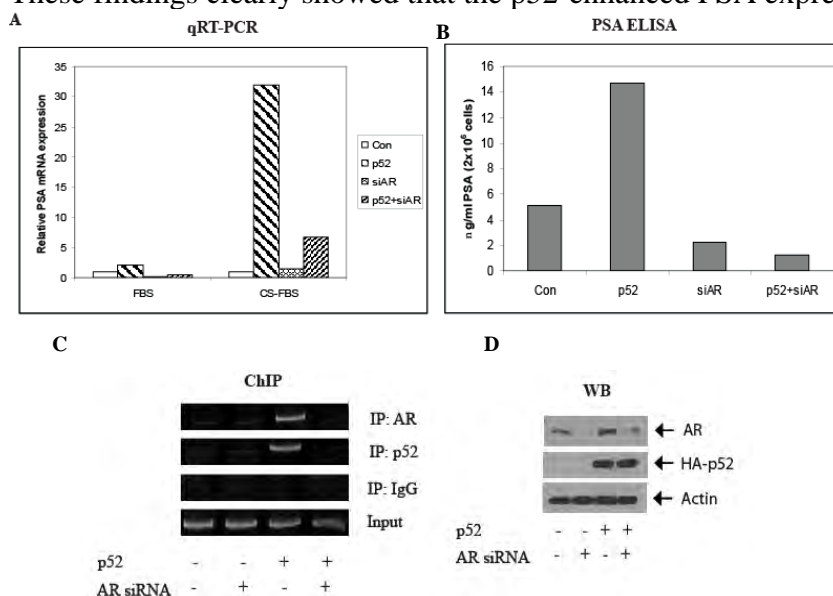


Fig. 7. p52-enhanced PSA expression and recruitment of p52 to AREIII are dependent on AR. LNCaP cells were transfected with plasmids expressing p52 or AR siRNA in FBS or CS-FBS. **A. qRT-PCR:** Real-time PCR analysis was performed for PSA mRNA. **B. PSA ELISA:** PSA protein expression was measured by ELISA in the supernatants. **C. ChIP:** ChIP assay was performed using AR, p52 or IgG antibodies. **D. WB:** Expression levels of AR and p52 in the above experiments.

NF- κ B2/p52 enhances the recruitment of p300 to the PSA distal enhancer.

Optimal activation of AR requires the recruitment of coregulators, including p300, SRC-1, or TIF-2, to the AREs. To test whether p52 enhances the recruitment of these coregulators, we transfected the pGL3-PSA-6.0-Luc reporter along with p52- and p300-

expressing plasmids or p300 siRNA into LNCaP cells in CS-FBS. Interestingly, pGL3-PSA-6.0-Luc was synergistically activated in the presence of p52 and p300 (>20-fold; Fig. 8A). p300 siRNA was able to reduce p52-induced transcriptional activity of AR. These results were confirmed using ChIP assays by transfecting p52 and p300 plasmids into LNCaP cells to test the recruitment of AR and p300 to AREIII. Overexpression of p52 enhanced the recruitment of p300 to AREIII, which was abolished by p300 siRNA (Fig. 8C). We also tested the effect of p300 siRNA on p52-mediated PSA mRNA expression using qRT-PCR. The results clearly showed that p300 siRNA almost completely abolished the p52-enhanced PSA expression (Fig. 8B). These results suggest that p52 may play an important role in the recruitment of coactivators such as p300 to AREs, thereby increasing the DNA-binding and, consequently, the transactivating ability of AR. LNCaP cells possess a functional but mutant AR. The expression levels of p300, AR and p52 were confirmed in the lysates (Fig. 8D).

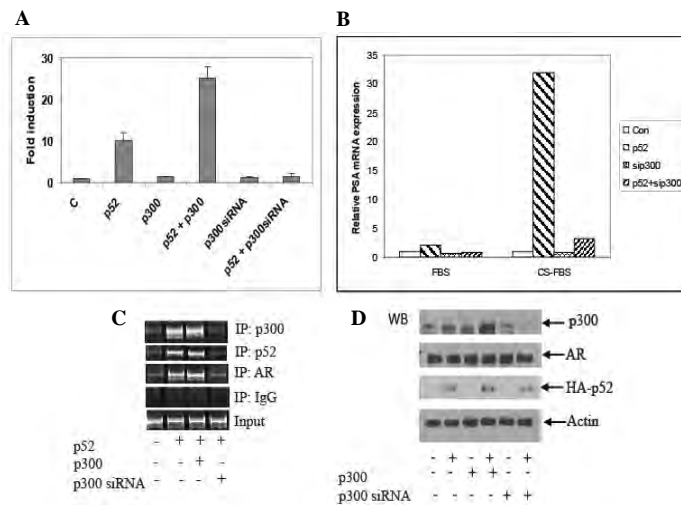


Fig. 8. p300 enhances PSA transactivation by p52. A. Androgen-deprived LNCaP cells were transfected with pGL3-PSA-Luc and plasmids expressing p52, p300, or p300 siRNA in CS-FBS for 48 h. Luciferase activities were measured and expressed as mean \pm SD of triplicate samples. B. p300 siRNA abolishes p52-induced increase in PSA mRNA. LNCaP cells transfected with plasmids expressing p52 or p300 siRNA were analyzed by qRT-PCR for PSA mRNA. C. p52 enhances p300 recruitment to the distal enhancer of PSA promoter. ChIP assay was performed using p300, p52, AR, or IgG antibodies and AREIII primers. D. WB: Expression levels of HA-p52, AR and p300 in the above experiments.

NF- κ B2/p52 induces recruitment of AR to AREs in NKX3.1 promoter.

To determine whether this effect is specific to PSA, we examined the effect of p52 expression on another typical AR target gene, NKX3.1. LNCaP and LAPC-4 cells were transfected with plasmids expressing p52, AR siRNA, or p300 siRNA in CSFBS. Total RNAs were analyzed by qRT-PCR for NKX3.1 mRNA. The results showed that p52 induced ~4-5-fold increase in NKX3.1 mRNA, which was abolished in the presence of AR or p300 siRNAs (Fig. 9A). ChIP assays were performed to confirm whether the observed induction of NKX3.1 transcription was mediated by recruitment of AR to AREs in its promoter. Results showed that p52 induced the recruitment of AR to AREs in NKX3.1 promoter in CS-FBS. Recruitment of p52 to the AREs was also observed, which was abolished by coexpression of AR siRNA in both LNCaP and LAPC-4 cells (Fig. 9B). The above findings showed that the effect of NF- κ B2/p52 in activation of AR is not restricted to PSA.

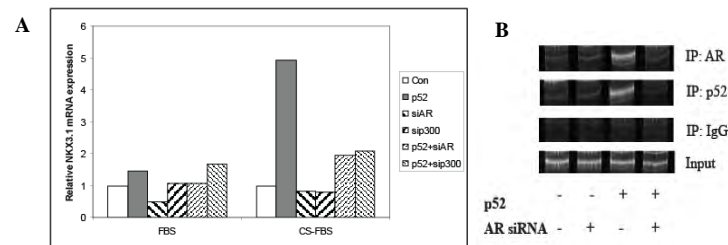


Fig. 9. A. p52 enhances NKX3.1 expression. LNCaP cells were transfected with the indicated plasmids and NKX3.1 expression was measured by qRT-PCR. B. p52 recruits AR to the NKX3.1 promoter. LNCaP cells were transfected with the indicated plasmids and ChIP assays were performed using primers spanning the ARE site in NKX3.1 promoter.

Downregulation of NF- κ B2/p52 in C4-2 cells abolishes AR activation.

C4-2 cells, a castration-resistant subline of LNCaP, exhibit high endogenous levels of p52 and constitutive activation of the AR compared to LNCaP. To test whether blocking endogenous p52 expression affects constitutive activation of AR in C4-2 cells, we transfected p52 and control short hairpin RNAs (shRNA) along with PSA-6.0-Luc reporter into C4-2 cells in FBS and CS-FBS. Luciferase activities were measured after downregulation of p52 expression was confirmed. The results showed that downregulation of p52 reduced transactivation of the PSA promoter-reporter by AR by ~80% (Fig. 10A). Total RNAs from the cells were also analyzed by qRT-PCR for PSA and NKX3.1 mRNAs. We found that downregulation of endogenous p52 expression resulted in 60% to 80% decrease in the transcript levels of both PSA and NKX3.1 (Fig. 10B & C, qRT-PCR). To test whether downregulation of p52 affects recruitment of AR to the promoters of PSA and NKX3.1 genes, ChIP assays were performed with extracts from C4-2 cells treated with p52 shRNA. Knockdown of p52 abolished the recruitment of AR to these promoters almost completely (Fig. 10D & E, ChIP). Expression levels of AR and p52 were verified by immunoblotting (Fig. 10F). These findings showed that downregulation of endogenous p52 expression reduced AR activity in cells expressing high levels of both proteins.

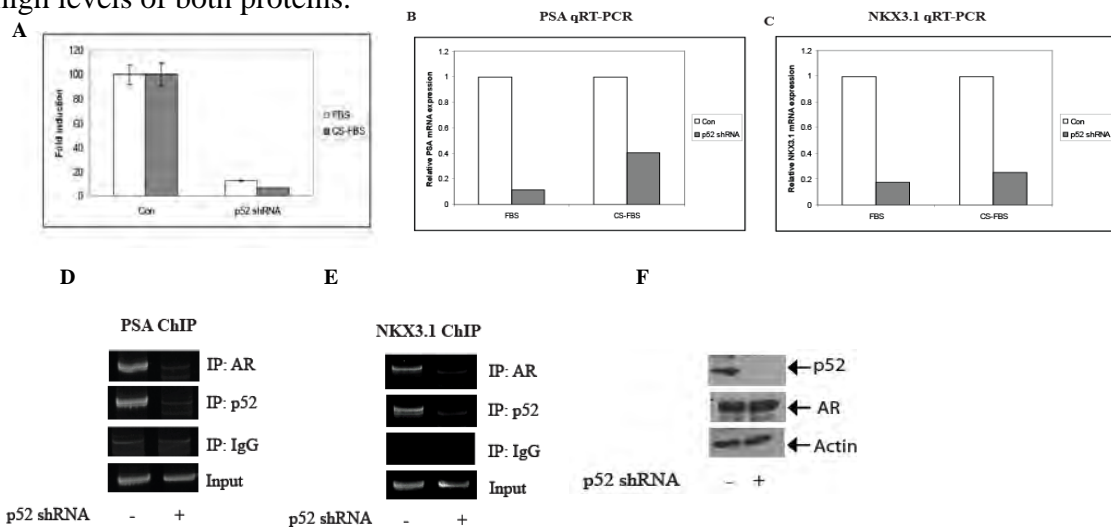


Fig. 10. Downregulation of endogenous p52 reduces AR activity in C4-2 cells. **A.** C4-2 cells were co-transfected with p52- or control-shRNAs and pGL3-PSA-Luc in FBS or CS-FBS and luciferase activity was measured after 48 h. Downregulation of endogenous p52 expression in C4-2 cells reduced the transactivation of PSA promoter. **B. PSA qRT-PCR:** Total RNAs were analyzed by qRT-PCR for PSA mRNA. **C. NKX3.1 qRT-PCR:** cDNAs were also analyzed for the expression of NKX3.1 mRNA by qRT-PCR. Results are expressed as fold change in expression. **D. PSA ChIP:** Extracts were analyzed by ChIP assay using PSA-AREIII primers. Recruitment of AR to AREIII was abolished when endogenous p52 expression was downregulated. **E. NKX3.1 ChIP:** Extracts were analyzed by ChIP assay using primers against NKX3.1-ARE site. Constitutive recruitment of AR to the ARE was dependent on endogenous p52 expression in C4-2 cells. **F.** Western blot showing downregulation of p52 expression by p52 shRNA in C4-2 cells.

3c. Determine whether p52 affects the DNA binding activity of AR.

To examine whether DNA binding activity of AR in androgen-depleted conditions is affected by p52, we transfected plasmid encoding p52 into LNCaP cells and prepared nuclear extracts. These nuclear extracts were used to perform Electrophoretic Mobility Shift Assays (EMSA) using a 32 P-labeled oligonucleotide containing AR consensus DNA binding site (Promega). Briefly, the AR consensus oligonucleotide was end labeled with

³²P-γ-ATP using T7 polynucleotide kinase. 10 μg of nuclear extracts were incubated with the labeled oligo in native reaction buffer for 30 min and the resulting complexes were resolved on 5% native PAGE. Bound complexes were visualized by autoradiography. The results showed that AR DNA-binding activity was enhanced in the presence of p52 compared to vector controls or parental LNCaP cells in CS-FBS (Fig. 11), suggesting that p52 induces AR DNA binding in the presence of castrate-levels of androgens.

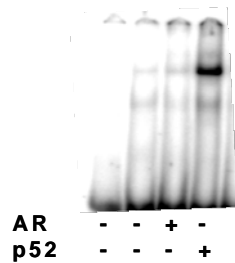


Fig. 11. LNCaP cells were transfected with AR or p52 expressing plasmids and EMSA assays were performed. Overexpression of p52 induced DNA-binding activity of endogenous AR in LNCaP cells.

p52 regulates expression of key genes involved in cellular processes

The gene expression profiles of LNCaP cells infected with empty vector and p52 encoding adenoviruses were analyzed using a high-throughput microarray containing >44,000 oligonucleotide-based gene probe sets (Agilent). These were mapped into the Gene-Spring software and were filtered according to manufacturer's instructions. Gene Ontology analysis was performed using Ingenuity Pathway Analysis Software (Ingenuity). The total number of genes differentially upregulated by >5-fold in LNCaP cells expressing p52 was ~130 (Table 2), whereas the total number of genes differentially downregulated by >2-fold was ~60 (Table 3). Among the potentially upregulated genes were genes belonging to functional categories (according to Ingenuity) like cell growth and proliferation, cell movement, cancer, cell cycle, cell signaling, etc. Among the genes found to be downregulated were: TP53, TP63, interferon gamma, the interleukins IL1A, IL2, and IL10, FAS and FAS ligand along with several others.

Key Research Accomplishments

We demonstrated that:

- Downregulation of p52 inhibits growth of prostate cancer cells
- Downregulation of p52 reduces expression of AR target genes like PSA and NKX3.1
- p52 induces nuclear translocation of AR in androgen-deprived conditions
- p52 induces AR DNA binding in a castration-resistant manner
- p52 induces recruitment of co-activators like p300 to the AR transcriptional complex in androgen-depleted conditions
- p52 activity is dependent on AR expression
- p52 regulates the expression of several genes involved in cell survival, angiogenesis and metastasis.

Reportable outcomes

Publications

1. Nadiminty N, Dutt S, Tepper C, Gao AC (2010) Microarray analysis reveals potential target genes of NF-kappaB2/p52 in LNCaP prostate cancer cells. *Prostate* 70(3): 276-87.
2. Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung HJ, Evans CP, Zhou Q, Gao AC (2010) Aberrant activation of the androgen receptor by NF-kappaB2/p52 in prostate cancer cells. *Cancer Res* 70(8): 3309-19.

Abstracts

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| 2010 | April, Aberrant activation of the androgen receptor by NF-kappaB2/p52, Annual Meeting, AACR, Washington DC. |
| 2010 | June, Aberrant activation of the androgen receptor by NF-kappaB2/p52, AUA Annual Meeting, San Francisco, CA. |

Conclusions

- We demonstrated that p52 activates AR signaling.
- p52 promotes castration-resistant growth of androgen sensitive LNCaP cells in vitro and in vivo.

References:

1. Nadiminty N, Chun JY, Lou W, Lin X, Gao AC (2009) NF-kappaB2/p52 enhances androgen-independent growth of human LNCaP cells via protection from apoptotic cell death and cell cycle arrest induced by androgen deprivation. *Prostate* 68(16): 1725-33.
2. Nadiminty N, Dutt S, Tepper C, Gao AC (2010) Microarray analysis reveals potential target genes of NF-kappaB2/p52 in LNCaP prostate cancer cells. *Prostate* 70(3): 276-87.
3. Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung HJ, Evans CP, Zhou Q, Gao AC (2010). Aberrant activation of the androgen receptor by NF-kappaB2/p52 in prostate cancer cells. *Cancer Res* 70(8): 3309-19.

Aberrant Activation of the Androgen Receptor by NF- κ B2/p52 in Prostate Cancer Cells

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Abstract

Prostate cancer initiation and progression are uniquely dependent on the androgen receptor (AR). Even when the cancer progresses to a castration-resistant stage, AR signaling remains active via a variety of mechanisms. In the present study, we showed that NF- κ B/p52 can activate the AR, resulting in increased transactivation of AR-responsive genes, such as *PSA* and *NKX3.1*, in a ligand-independent manner. NF- κ B2/p52 enhances nuclear translocation and activation of AR by interacting with its NH₂-terminal domain and enhances the recruitment of coactivators such as p300 to the promoters of AR-dependent genes. These results were confirmed in three different prostate cancer cell lines: LAPC-4 (wild-type AR), LNCaP (mutant AR), and C4-2 (castration resistant). Transfection of p52 into LAPC-4 and LNCaP cells (which express low levels of p52) showed increased activation of the endogenous AR. Downregulation of endogenous p52 in C4-2 cells resulted in abrogation of AR constitutive activation. Comparison of the relative effects of p52 and p65 (RelA) showed that p52, but not p65, could activate the AR. Collectively, these findings, together with previous reports that the levels of NF- κ B2/p52 are elevated in prostate cancer cells and that active NF- κ B2/p52 promotes prostate cancer cell growth *in vitro* and *in vivo*, suggest that NF- κ B2/p52 may play a critical role in the progression of castration-resistant prostate cancer. *Cancer Res*; 70(8); 3309–19. ©2010 AACR.

Introduction

As the growth of prostate cancer cells depends on the presence of androgens, almost all patients respond initially to androgen ablation. However, virtually every patient will relapse due to the growth of castration-resistant cancer cells. Androgen receptor (AR) activation plays an important role in prostate carcinogenesis and progression. Ligand-dependent activation leads to the binding of AR to androgen response elements (ARE). Castration-resistant prostate cancer (CRPC) cells often continue to express androgen-responsive genes, such as *prostate-specific antigen (PSA)*, and often express AR (1, 2), suggesting that the AR becomes activated by an androgen-independent mechanism in AR-positive CRPC cells. The AR can also be activated in the absence of or very low levels of androgen by cross talk with other signaling pathways (3–9). Mutations, deletions, and amplification of

the *AR* gene or alterations of the interactions between AR and some of its coregulators may increase tumor cell sensitivity to very low levels of androgen or allow it to respond to other steroids or even antiandrogens (10–14). In addition, levels of intraprostatic androgens are at concentrations sufficient to activate the AR and stimulate tumor growth (15–18). Thus, abnormal activation of the AR plays a major role in the development of castration resistance.

The NF- κ B family comprises five proteins—RelA/p65, NF- κ B1/p50, c-Rel, RelB, and NF- κ B2/p52—which have been identified as important mediators in the oncogenesis of many cancers. The classic NF- κ B pathway involving the p65/p50 heterodimer has been well studied and shown to be constitutively activated in several cancers, including prostate. The noncanonical NF- κ B pathway involves the processing of p100 to NF- κ B2/p52 via the recruitment of NF- κ B-inducing kinase and subsequent activation of I κ B kinase α . The processing of p100 to p52 is a tightly controlled event in many cells and tissues (19–22). The proteolytic processing of p100 to p52 can be activated by lymphotoxin β (23, 24), B-cell-activating factor (25, 26), CD40 ligand (24, 27), and its *cis*-acting domain (22). The functional significance of p100 processing has been confirmed by genetic evidence from humans and mice (28). Constitutive production of p52 in p100 knock-in mice causes marked hyperplasia in lymphocytes and liver, leading to early postnatal death (27). Overproduction of p52 in the mammary gland in p100 transgenic mice disrupted normal ductal development and led to hyperplastic growth (29). Overproduction of p52 has been observed in several solid tumors, including breast and prostate

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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cancers (30, 31). We previously showed that p100 is processed to p52 by activated signal transducer and activator of transcription 3 (Stat3) in prostate cancer cells (32). Further studies showed that NF- κ B2/p52 induces castration-resistant growth in LNCaP cells by inhibiting cell cycle arrest and apoptotic cell death induced by androgen deprivation (33). This was accompanied by continued expression and activation of the AR, which suggests that p52 may activate AR during the progression of CRPC. Our studies also show that several genes involved in cell growth, proliferation, cell movement, etc. are potential targets of NF- κ B2/p52 (34).

In this study, we examined the mechanism of AR activation by p52. We show that AR is activated by p52 via recruiting the AR and coactivators such as p300 to the promoters of AR-responsive genes, resulting in their increased transactivation even in androgen-depleted conditions.

Materials and Methods

Cells, reagents, and antibodies. LAPC-4, LNCaP, C4-2, and PC-3 prostate cancer cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 containing either 10% complete fetal bovine serum (FBS) or 10% charcoal-dextran-stripped FBS (CS-FBS) and penicillin/streptomycin. LNCaP passage numbers <20 were used throughout the study. NF- κ B2/p52 (K-27), AR (441; mouse monoclonal), hemagglutinin (HA), actin, and tubulin antibodies were purchased from Santa Cruz Biotechnology. Anti-Flag-M2 antibodies were purchased from Sigma Chemical Co. All other reagents were of analytic grade and obtained from local suppliers.

Western blot analysis. Cells were lysed in high salt buffer as described earlier (33). Western blots were probed with the indicated primary antibodies, and the chemiluminescence was detected by ECL (Amersham) after incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies.

Measurement of PSA. PSA levels were measured in the culture supernatants using ELISA (United Biotech, Inc.) according to the manufacturer's instructions and as described previously (35).

Luciferase assays. LAPC-4 and LNCaP cells were transfected with pGL3-ARE-Luc, pGL3-PSA6.0-Luc, pGL3-AREI/II-Luc, and pGL3-AREIII-Luc reporters along with p52, p65, p100, AR, p300 plasmids, and AR and p300 siRNAs as indicated in the figures (see Supplementary Materials and Methods for details). Cell lysates were subjected to luciferase assays with the Luciferase Assay System (Promega).

Coimmunoprecipitation. Equal amounts of LNCaP or C4-2 cell lysates were immunoprecipitated with 1 μ g of anti-AR, anti-Flag, anti-p52, or anti-HA antibodies (as indicated in the figures) overnight. The precipitated proteins were analyzed by Western blotting with the indicated antibodies.

Chromatin immunoprecipitation assay. LAPC-4 and LNCaP cells were transfected with the indicated plasmids, and 1 nmol/L dihydrotestosterone (DHT) was added 24 h after transfection. DNA-protein complexes were isolated, and chromatin immunoprecipitation (ChIP) assays were per-

formed as described earlier using primers spanning either the proximal or the distal enhancer AREs of the PSA promoter or ARE sites in the NKX3.1 promoter (−3013 to −2995; refs. 36, 37). Isotype-matched IgG was used as control (primer sequences in Supplementary Materials and Methods).

Immunofluorescence. LNCaP (grown in CS-FBS for 3 d to reduce background AR activation) and PC-3 cells were transfected with the indicated plasmids or treated with 1 nmol/L DHT and used to assess the extent of AR nuclear translocation by immunofluorescence (see Supplementary Materials and Methods for details).

Real-time quantitative reverse transcription-PCR. LNCaP and LAPC-4 cells were transfected with the indicated plasmids, and total RNAs were extracted using Trizol reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription-PCR (RT-PCR) using SYBR Green iQ Supermix (Bio-Rad) according to the manufacturer's instructions. Each reaction was normalized by coamplification of actin. Triplicates of samples were run on default settings of the real-time PCR machine (Bio-Rad) as described earlier (primer sequences in Supplementary Materials and Methods; ref. 38).

Statistical analysis. Data are shown as the mean \pm SD. Multiple group comparison was performed by one-way ANOVA followed by the Scheffe procedure for comparison of means. $P < 0.05$ was considered significant.

Results

NF- κ B2/p52 enhances PSA expression. Our previous studies found that LNCaP cells overexpressing NF- κ B2/p52 were able to form rapidly growing tumors when injected s.c. into castrated nude mice. This was accompanied by continued expression and activation of the AR as well as production of PSA in castrated animals (33). To verify that p52 enhances PSA expression *in vitro*, LNCaP cells transfected with varying amounts of p52-expressing plasmids were grown in CS-FBS. Secretion of PSA was assayed in the supernatants by ELISA after 48 hours. The values were normalized to cell numbers. Expression of p52 induced a dose-dependent increase in PSA expression in the absence of androgen in LNCaP cells. This result was also confirmed by real-time RT-PCR to measure endogenous PSA mRNA expression. Expression of p52 enhanced PSA mRNA expression ~30-fold in CS-FBS (Fig. 1A). These observations confirm the *in vivo* finding that mice bearing LNCaP tumors expressing p52 have elevated PSA levels.

NF- κ B2/p52 induces transactivation of PSA promoter. We used PSA promoter as a model to examine the effects of p52 on AR signaling, as it is the best-characterized androgen-responsive promoter. LNCaP cells were cotransfected with PSA enhancer-promoter-luciferase (pGL3-PSA-6.0-Luc) and plasmids carrying p52, p65, p100, or vector control. As shown in Fig. 1B, PSA promoter activity was increased 11-fold by overexpression of p52 compared with the vector control in CS-FBS. However, p100 decreased PSA promoter activity by ~2-fold compared with the vector control. In contrast to the activation of AR by p52, overexpression of p65

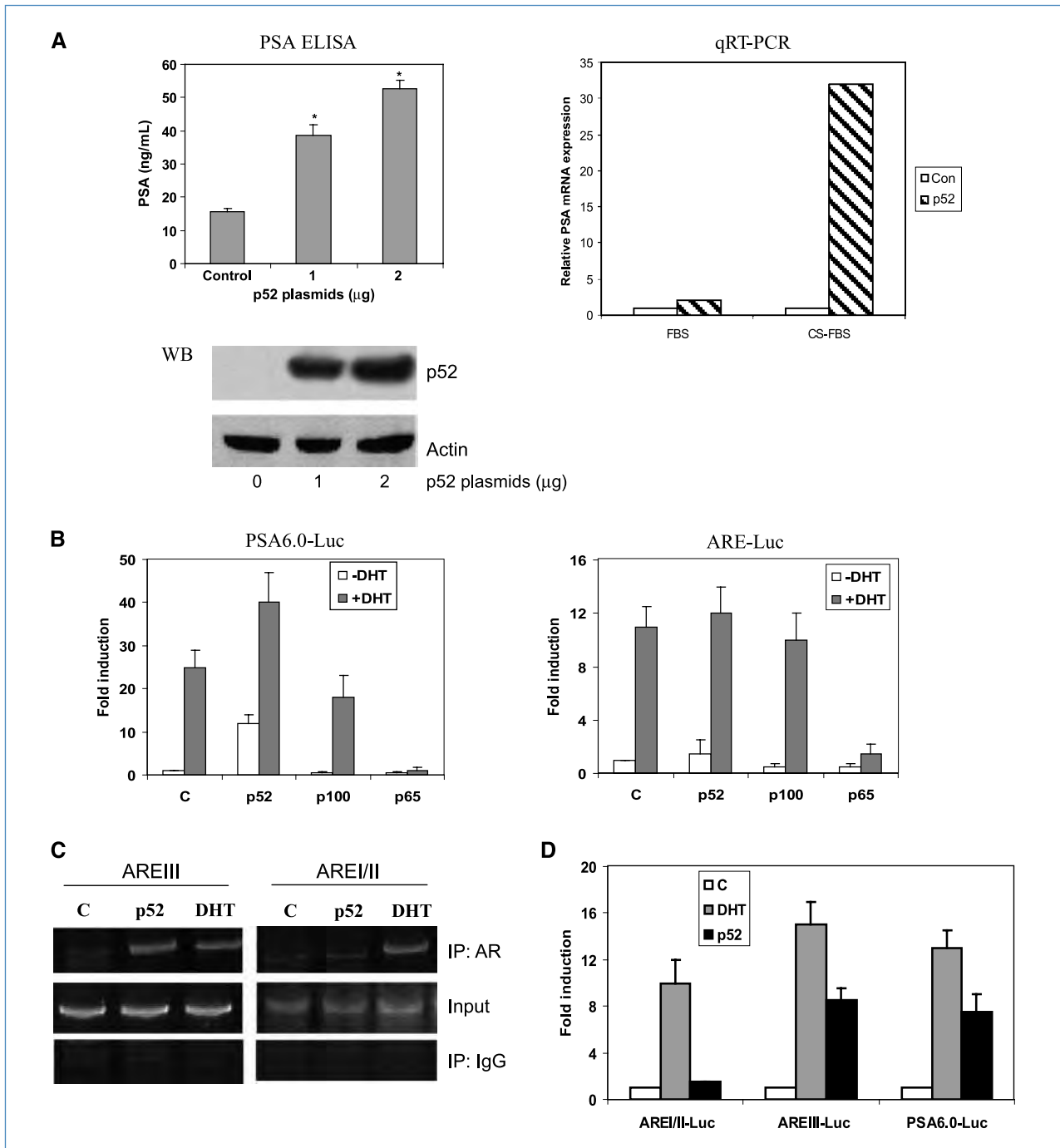


Figure 1. A, p52 enhances PSA expression. LNCaP cells were transfected with 1 to 2 μ g of p52 plasmid in CS-FBS for 2 d. PSA ELISA: PSA secretion was measured by ELISA in supernatants. Columns, mean of triplicate samples; bars, SD. *, $P < 0.05$. Western blot (WB): cell lysates were analyzed by immunoblotting using p52 antibodies. Actin was the internal control. qRT-PCR: total RNAs from p52-transfected and vector control-transfected LNCaP cells were analyzed by qRT-PCR for PSA mRNA expression. p52-induced PSA mRNA levels are expressed relative to their respective controls in FBS and CS-FBS. B, p52 transactivates PSA promoter. LNCaP cells in CS-FBS were cotransfected with pGL3-PSA6.0-Luc or ARE-Luc and plasmids expressing p52, p100, or p65. Cells were cultured in CS-FBS (–DHT) or 1 nmol/L DHT (+DHT) for 48 h. Luciferase activities were measured. Columns, mean of triplicate samples; bars, SD. Three independent experiments were performed. C, p52 recruits AR preferentially to the distal enhancer of PSA promoter. Androgen-deprived LNCaP cells were transfected with plasmids expressing p52 and vector \pm 1 nmol/L DHT. ChIP assays were performed using AR antibodies. p52-activated AR is recruited only to AREIII, whereas DHT-activated AR is recruited to both AREIII and ARE/II. D, androgen-deprived LNCaP cells were cotransfected with p52 and pGL3-ARE/II-Luc, pGL3-AREIII-Luc, or pGL3-PSA-Luc \pm 1 nmol/L DHT for 48 h. Luciferase activities were measured. Columns, mean of triplicate samples; bars, SD. Three independent experiments were performed.

significantly reduced DHT-induced transactivation of ARE-Luc and pGL3-PSA-6.0-Luc reporters, consistent with previous reports that NF- κ B-p52 and AR exhibit mutual transcriptional antagonism (39, 40). To our surprise, p52 did not induce ARE-Luc activity, which can be activated by DHT (Fig. 1B), suggesting that p52-activated AR is distinct from androgen-bound AR.

NF- κ B2/p52-activated AR is preferentially recruited to the PSA distal enhancer. The PSA promoter in the PSA-Luc construct contains two sets of AREs: AREI/II (the proximal enhancer) located at -0.1 kb and AREIII (the distal enhancer) located at ~ -6 kb. The ARE-Luc construct contains only AREI/II (the proximal enhancer). To determine whether p52 modulates recruitment of AR to the PSA promoter, ChIP assays were performed to examine the recruitment of AR by p52 and DHT. As shown in Fig. 1C, p52-activated AR binds only to the distal enhancer (AREIII), whereas DHT-bound AR is recruited to both ARE sites. To test whether the differential recruitment of AR to AREs by p52 translates to differential AR activation, we performed luciferase assays using reporters containing the enhancer element (AREIII-Luc), the proximal AREs (AREI/II-Luc), or the full-length PSA promoter (PSA-6.0-Luc). We found that p52 induced the activity of AREIII-Luc and the full-length promoter but not AREI/II-Luc. As expected, DHT stimulates the activity of AREI/II-Luc, AREIII-Luc, and PSA-6.0-Luc (Fig. 1D). These data showed that p52-activated AR is preferentially recruited to the PSA distal enhancer.

NF- κ B2/p52-induced PSA expression is dependent on AR expression. Previous studies showed that NF- κ B-p52/p50 complex binds directly to the distal core enhancer region in the PSA promoter and enhances PSA expression (41). So, we examined whether p52 binding to the AREIII element in the PSA enhancer is AR dependent or independent. Total RNAs were isolated from LNCaP cells transfected with plasmids encoding p52 and AR siRNA, and PSA mRNA expression was analyzed by quantitative RT-PCR (qRT-PCR). We found that p52 enhanced PSA mRNA expression ~ 30 -fold in CS-FBS, which was almost completely abolished by downregulation of AR. This was also confirmed using ELISA, where p52 induced the secretion of PSA by LNCaP cells in CS-FBS, which was abolished by downregulation of AR (Fig. 2A). To confirm that the recruitment of p52 to AREIII is AR dependent, we performed ChIP assay with lysates from LNCaP cells transfected with p52 and AR siRNA in CS-FBS. The results showed that recruitment of p52 to the AREIII site was completely AR dependent (Fig. 2A, ChIP), which was consistent with the above results. Lysates from these cells were analyzed by immunoblotting with anti-AR antibodies to confirm that AR expression was downregulated $>70\%$ in AR siRNA-transfected cells (Fig. 2A, WB). These findings clearly showed that the p52-enhanced PSA expression is AR dependent.

NF- κ B2/p52 enhances the recruitment of p300 to the PSA distal enhancer. Optimal activation of AR requires the recruitment of coregulators, including p300, SRC-1, or TIF-2, to the AREs. To test whether p52 enhances the recruitment of these coregulators, we transfected the pGL3-PSA-6.0-Luc reporter along with p52- and p300-expressing plasmids or

p300 siRNA into LNCaP cells in CS-FBS. Interestingly, pGL3-PSA-6.0-Luc was synergistically activated in the presence of p52 and p300 (>20 -fold; Fig. 2B). p300 siRNA was able to reduce p52-induced transcriptional activity of AR. These results were confirmed using ChIP assays by transfecting p52 and p300 plasmids into LNCaP cells to test the recruitment of AR and p300 to AREIII. Overexpression of p52 enhanced the recruitment of p300 to AREIII, which was abolished by p300 siRNA (Fig. 2C). We also tested the effect of p300 siRNA on p52-mediated PSA mRNA expression using qRT-PCR. The results clearly showed that p300 siRNA almost completely abolished the p52-enhanced PSA expression (Fig. 2D). These results suggest that p52 may play an important role in the recruitment of coactivators such as p300 to AREs, thereby increasing the DNA-binding and, consequently, the transactivating ability of AR. LNCaP cells possess a functional but mutant AR. To test whether wild-type AR (Wt-AR) responds similarly to p52 expression, the experiments were repeated in LAPC-4 cells, which possess Wt-AR with similar results (Supplementary Fig. S1). These findings confirm that mutant AR in LNCaP cells as well as Wt-AR in LAPC-4 cells responds to activation by p52 in a similar manner.

NF- κ B2/p52 enhances the nuclear translocation of AR. To test whether the above effects were due to an increase in p52-induced AR expression, LNCaP cells were grown in CS-FBS for 2 days and transfected with p52-expressing plasmids. Whole-cell extracts were analyzed with anti-AR antibodies. The results showed that p52 did not enhance the expression of AR (Fig. 3A). The AR typically translocates from the cytoplasm to the nucleus on activation. To examine whether p52 enhances nuclear translocation of the AR protein in the presence or absence of low levels of androgen, nuclear extracts from p52-transfected LNCaP cells grown in CS-FBS were analyzed by immunoblotting with anti-AR antibodies. The level of nuclear translocation of AR under conditions of androgen deprivation was very low, which was enhanced with the expression of p52 (Fig. 3B). To further confirm these results, LNCaP cells transfected with p52 or control were processed for immunofluorescent staining with AR antibodies. AR was confined mainly to the cytoplasmic compartment in control cells in CS-FBS, whereas stronger nuclear staining of AR was observed in p52-transfected cells, although some AR was still seen in the cytoplasm (Fig. 3C, top). To verify that these results were not due to the T877A mutation present in the AR in LNCaP cells, we cotransfected AR-negative PC-3 cells with Wt-AR and p52 followed by immunofluorescent staining with AR antibodies. AR expression was mostly seen in the cytoplasmic compartment in control cells, whereas it was mostly located in the nucleus in p52-transfected cells (Fig. 3C, bottom). These results suggested that p52 may facilitate the nuclear translocation of AR during androgen deprivation.

NF- κ B2/p52 interacts with the NH₂-terminal domain of AR. The above results suggested a physical interaction between the AR and p52. To determine whether p52 can interact with the AR, we transfected HA-p52 into LNCaP cells along with Wt-AR. Nuclear fractions were immunoprecipitated with anti-p52 antibodies, and the resulting eluates were analyzed

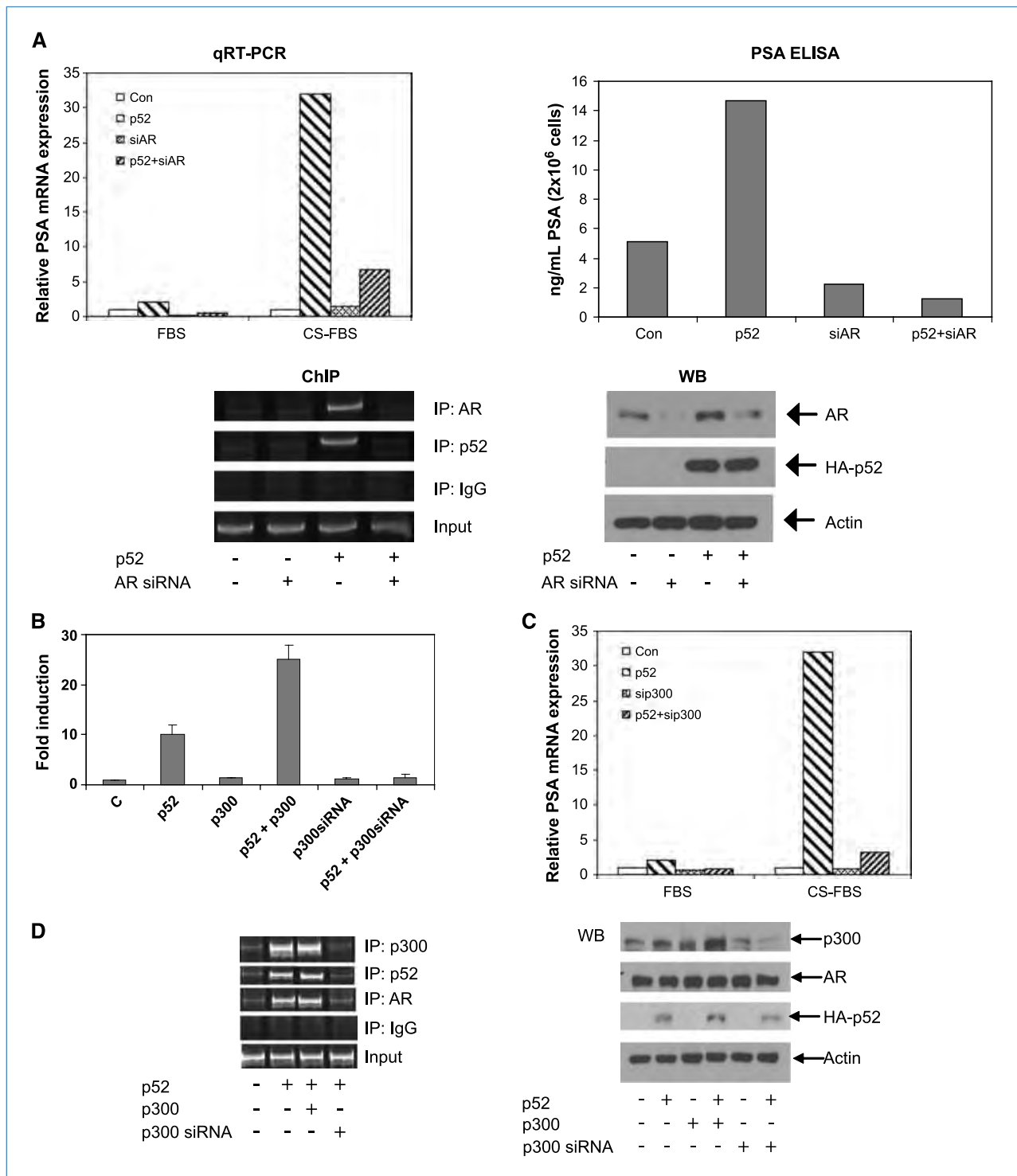


Figure 2. p52-enhanced PSA expression is dependent on AR. LNCaP cells were transfected with plasmids expressing p52 or AR siRNA in FBS or CS-FBS. A, qRT-PCR: real-time PCR analysis was performed for PSA mRNA and results were expressed as fold change in relative mRNA expression. PSA ELISA: PSA protein expression was measured by ELISA in the supernatants. ChIP: ChIP assay was performed using AR, p52, or IgG antibodies. Recruitment of p52 to AREIII in the PSA promoter was abolished in the absence of AR. WB: expression levels of AR and p52 in the above experiments. B, p300 enhances PSA transactivation by p52. Androgen-deprived LNCaP cells were transfected with pGL3-PSA-Luc and plasmids expressing p52, p300, or p300 siRNA in CS-FBS for 48 h. Luciferase activities were measured. Columns, mean of triplicate samples; bars, SD. Three independent experiments were performed. C, p300 siRNA abolishes p52-induced increase in PSA mRNA. LNCaP cells transfected with plasmids expressing p52 or p300 siRNA were analyzed by qRT-PCR for PSA mRNA. D, p52 enhances p300 recruitment to the distal enhancer of PSA promoter. ChIP assay was performed using p300, p52, AR, or IgG antibodies and AREIII primers. WB: expression levels of HA-p52, AR, and p300 in the above experiments.

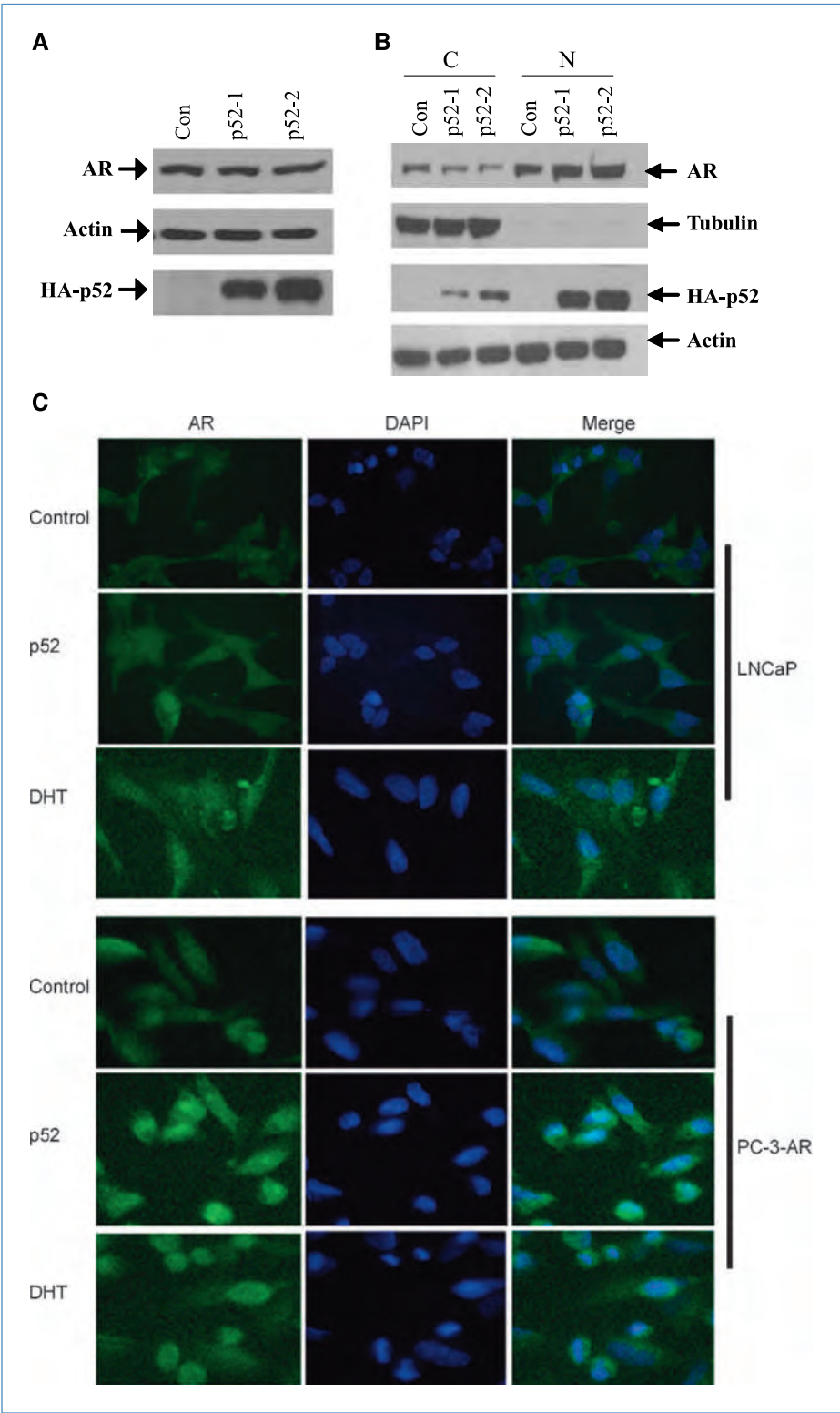


Figure 3. p52 induces nuclear translocation of AR. Androgen-deprived LNCaP cells were transfected with HA-p52 and control vector. Whole-cell (A), cytoplasmic (C), and nuclear (N) proteins (B) were immunoblotted with AR, actin, tubulin, or HA antibodies. Actin and tubulin were used as loading controls for whole-cell and cytoplasmic proteins, respectively. C, top, androgen-deprived LNCaP cells were transfected with p52 plasmid (1 or 2 μ g) or control vector or treated with 1 nmol/L DHT for 24 h; bottom, PC-3 cells were transfected with Wt-AR and HA-p52 in CS-FBS for 24 h. Cells were processed for immunofluorescent staining of AR, and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

by immunoblotting with anti-AR antibodies. We observed that p52 was able to coimmunoprecipitate with AR in the nuclear extracts (Fig. 4A). Immunoprecipitation with anti-p52 antibodies did not pull down detectable levels of AR in

p52-nontransfected cells due to low levels of p52 in LNCaP cells (33). Coimmunoprecipitation of endogenous p52 with endogenous AR was also observed in C4-2 cells (Fig. 4B). These results suggest that p52 physically interacts with AR.

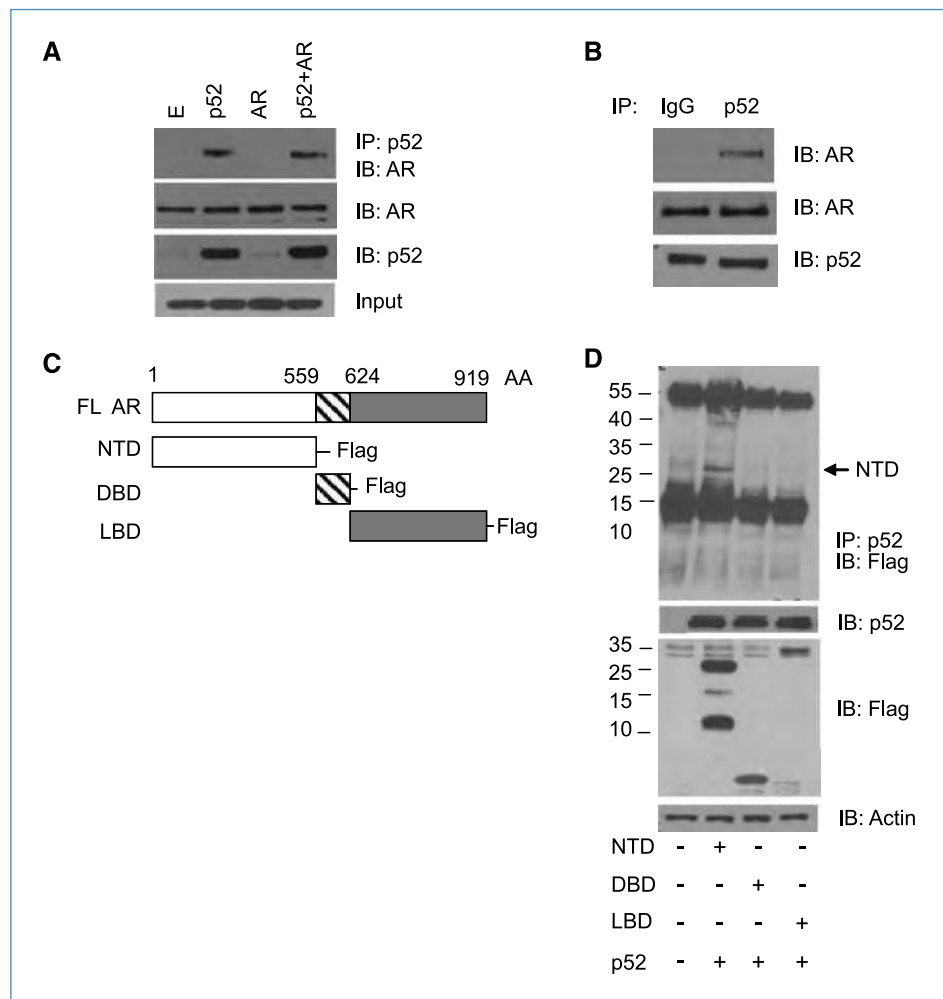
To test which domain of the AR interacts with p52, we cloned the three domains of AR [NH₂-terminal transactivation domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD)] into Flag-tagged expression vectors by PCR amplification (Fig. 4C; Supplementary Fig. S2A; Supplementary Results). LNCaP cells were transfected with HA-tagged p52 and Flag-tagged AR-NTD/DBD/LBD-expressing plasmids and immunoprecipitated with anti-p52 antibodies and immunoblotted with anti-Flag antibodies (Fig. 4D) or immunoprecipitated with anti-Flag antibodies and probed with anti-HA antibodies (Supplementary Fig. S2B). These experiments revealed that the NTD of AR coprecipitates with p52, suggesting that p52 may interact with the NTD of AR.

NF- κ B2/p52 induces recruitment of AR to AREs in NKX3.1 promoter. The above results showed that NF- κ B2/p52 activates transcription of the androgen-responsive gene *PSA* under conditions of androgen deprivation. To determine whether this effect is specific to PSA, we examined the effect of p52 expression on another typical AR target gene, *NKX3.1*. LNCaP and LAPC-4 cells were transfected with

plasmids expressing p52, AR siRNA, or p300 siRNA in CS-FBS. Total RNAs were analyzed by qRT-PCR for *NKX3.1* mRNA. The results showed that p52 induced ~4-5-fold increase in *NKX3.1* mRNA, which was abolished in the presence of AR or p300 siRNAs (Fig. 5A and C). ChIP assays were performed to confirm whether the observed induction of *NKX3.1* transcription was mediated by recruitment of AR to AREs in its promoter. Results showed that p52 induced the recruitment of AR to AREs in *NKX3.1* promoter in CS-FBS. Recruitment of p52 to the AREs was also observed, which was abolished by coexpression of AR siRNA in both LNCaP and LAPC-4 cells (Fig. 5B and D). The above findings showed that the effect of NF- κ B2/p52 in activation of AR is not restricted to PSA.

Downregulation of NF- κ B2/p52 in C4-2 cells abolishes AR activation. C4-2 cells, a castration-resistant subline of LNCaP, exhibit high endogenous levels of p52 and constitutive activation of the AR compared with LNCaP. To test whether blocking endogenous p52 expression affects constitutive activation of AR in C4-2 cells, we transfected p52 and control short hairpin RNAs (shRNA) along with

Figure 4. p52 interacts with the NTD of AR. A, p52 immunoprecipitates with AR. Nuclear extracts from LNCaP cells were immunoprecipitated with p52 antibodies and probed for AR. Bottom, expression of AR or p52 in nuclear extracts. B, endogenous p52 immunoprecipitates with AR in C4-2 cells. Nuclear extracts from C4-2 cells were immunoprecipitated (IP) with p52 or IgG antibodies and immunoblotted (IB) with AR antibodies. C, schematic representation of the functional domains of AR [919 amino acids (AA)]. D, p52 associates with the NTD of AR. Extracts from LNCaP cells expressing Flag-tagged DBD, LBD, and NTD and p52 were immunoprecipitated with p52 antibodies and probed with anti-Flag antibodies. Bottom, immunoblotting with p52, Flag, and actin antibodies in cell extracts.



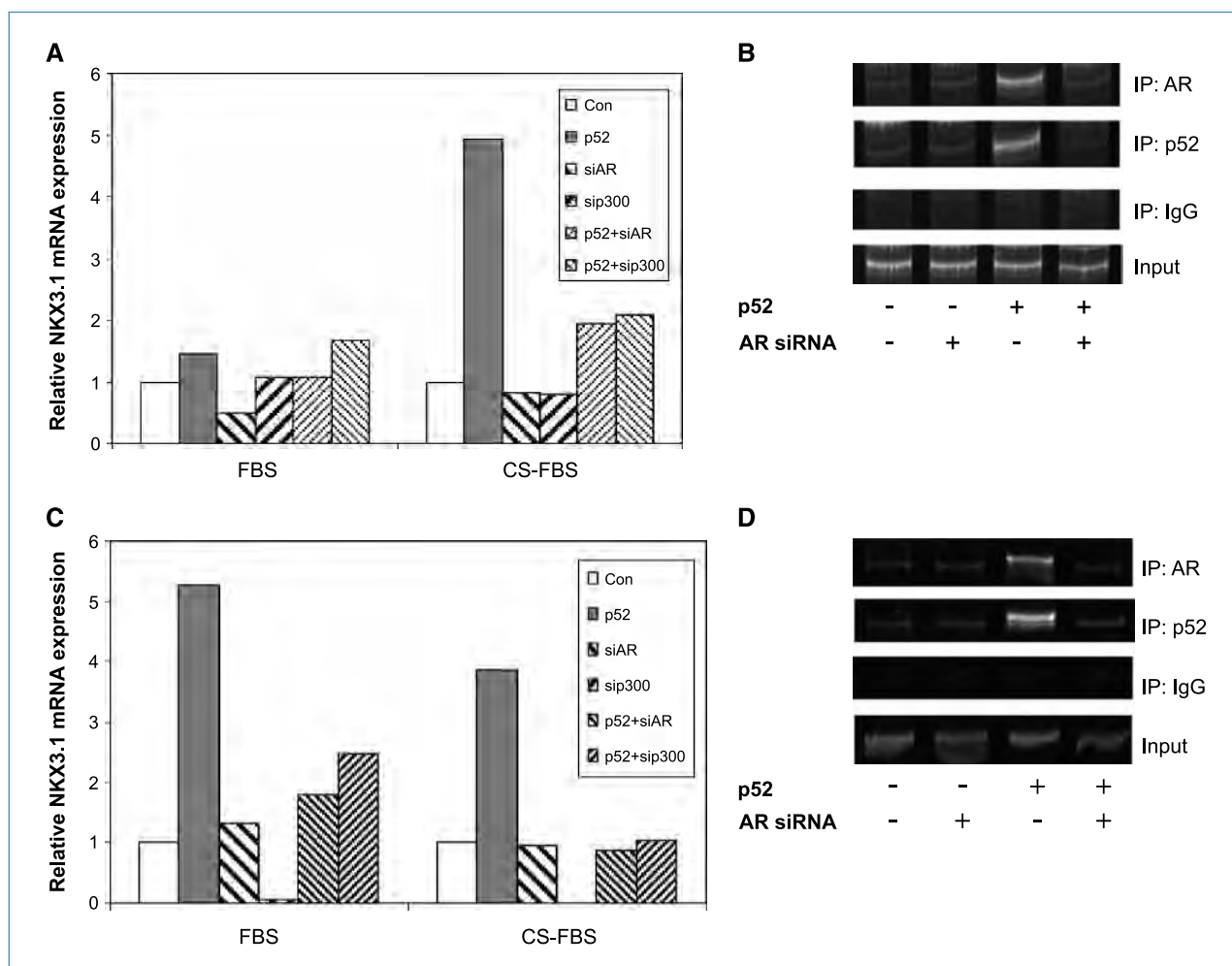


Figure 5. Expression of the AR-regulated gene *NKX3.1* is enhanced by p52 in an AR-dependent manner. LNCaP cells were transfected with plasmids expressing p52 (1 μ g), or p300 and AR siRNAs in FBS or CS-FBS. A, total RNAs were analyzed by qRT-PCR for *NKX3.1* mRNA. p52 induced the expression of *NKX3.1* mRNA, which was abolished by downregulation of AR. B, ChIP assays were performed with AR, p52, or IgG antibodies. p52 enhanced the recruitment of AR to *NKX3.1* promoter, whereas recruitment of p52 was AR dependent. LAPC-4 cells were transfected with plasmids expressing p52, or p300 or AR siRNAs in FBS or CS-FBS. C, total RNAs were analyzed by qRT-PCR for *NKX3.1* mRNA. p52-induced expression of *NKX3.1* mRNA was abolished by downregulation of AR. D, ChIP assays were performed with AR, p52, or IgG antibodies. p52 induced the recruitment of AR to *NKX3.1* promoter, whereas recruitment of p52 was AR dependent.

PSA-6.0-Luc reporter into C4-2 cells in FBS and CS-FBS. Luciferase activities were measured after downregulation of p52 expression was confirmed. The results showed that downregulation of p52 reduced transactivation of the PSA promoter-reporter by AR by ~80% (Fig. 6A). Total RNAs from the cells were also analyzed by qRT-PCR for PSA and *NKX3.1* mRNAs. We found that downregulation of endogenous p52 expression resulted in 60% to 80% decrease in the transcript levels of both PSA and *NKX3.1* (Fig. 6B and C, qRT-PCR). To test whether downregulation of p52 affects recruitment of AR to the promoters of *PSA* and *NKX3.1* genes, ChIP assays were performed with extracts from C4-2 cells treated with p52 shRNA. Knockdown of p52 abolished the recruitment of AR to these promoters almost completely (Fig. 6B and C, ChIP). Expression levels of AR and p52 were verified by immunoblotting (Fig. 6D). These findings showed that downregulation of

endogenous p52 expression reduced AR activity in cells expressing high levels of both proteins.

Comparison of relative effects of p52 and p65 on AR. To determine whether the above observed effects of p52 on AR activation were due to a p52-induced increase in AR expression and to compare the relative effects of p52 and p65 on AR expression, we analyzed AR protein and mRNA levels in LNCaP and LAPC-4 cells transfected with p52 or p65 by immunoblotting and qRT-PCR. Although we observed 1.5- to 2-fold induction of AR mRNA levels by p52, no significant induction in protein levels was detected by overexpression of p52 or p65 (Supplementary Fig. S3A and C). However, p52, but not p65, induced AR nuclear translocation (Fig. 3; Supplementary Fig. S3B).

To compare the effects of p52 and p65 on AR-regulated genes, we analyzed expression levels of PSA and *NKX3.1*

in LNCaP and LAPC-4 cells expressing p52 or p65 in the presence or absence of androgen. p52, and not p65, induced significant increases in expression levels of PSA and NKX3.1 (Supplementary Fig. S4A–C) and transacti-

vated PSA promoter-driven luciferase reporter (Supplementary Fig. S4D). These findings showed that p52-induced AR activation is not due to an increase in AR expression.

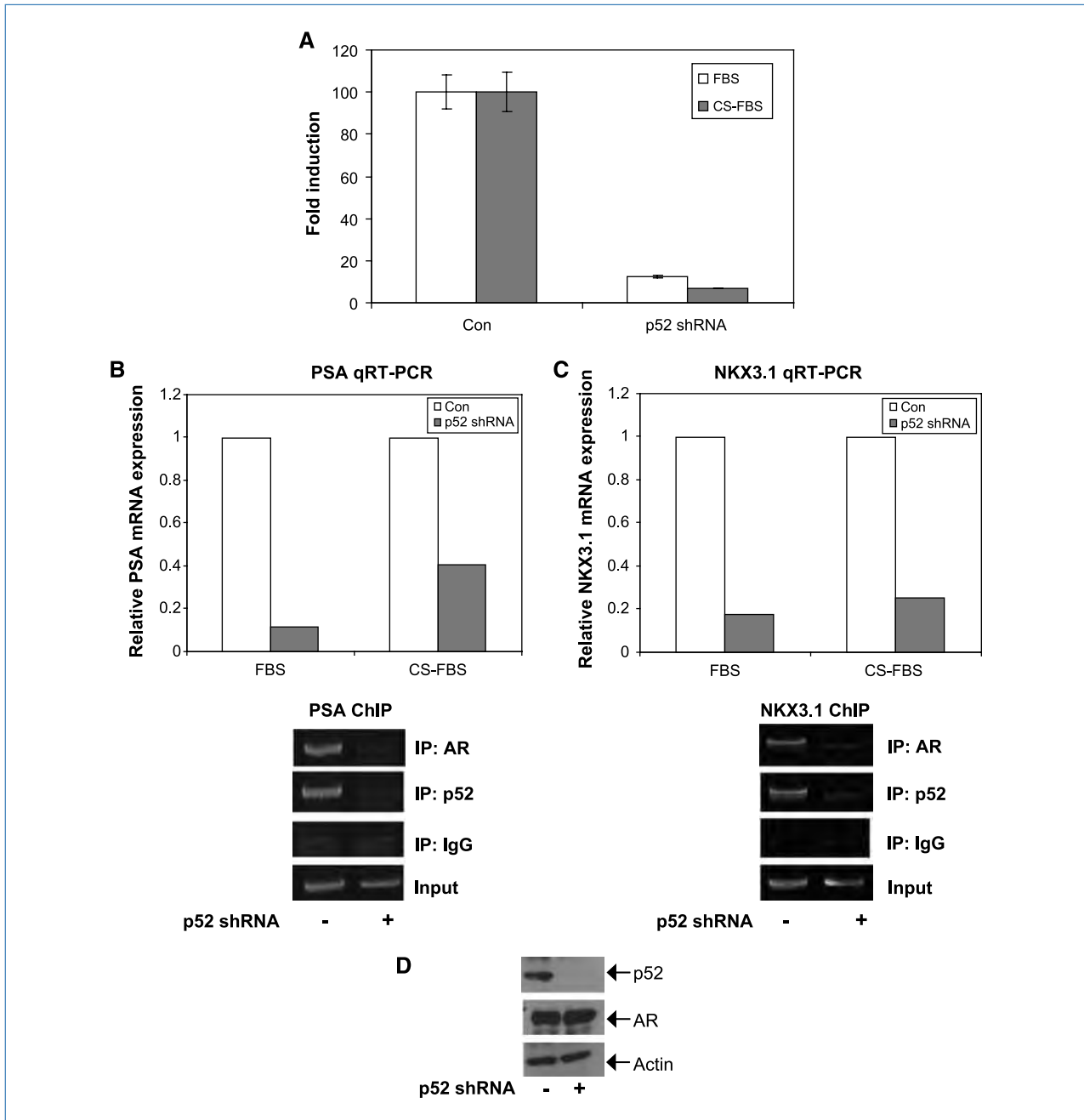


Figure 6. Downregulation of endogenous p52 reduces AR activity in C4-2 cells. A, C4-2 cells were cotransfected with p52 or control shRNAs and pGL3-PSA-Luc in FBS or CS-FBS, and luciferase activity was measured after 48 h. Columns, mean; bars, SD. Downregulation of endogenous p52 expression in C4-2 cells reduced the transactivation of PSA promoter. C4-2 cells were transfected with p52 or control shRNAs. B, PSA qRT-PCR: total RNAs were analyzed by qRT-PCR for PSA mRNA. Results are expressed as fold change in expression. PSA ChIP: extracts were analyzed by ChIP assay using PSA-AREIII primers. Recruitment of AR to AREIII was abolished when endogenous p52 expression was downregulated. C, NKX3.1 qRT-PCR: cDNAs were also analyzed for the expression of NKX3.1 mRNA by qRT-PCR. Results are expressed as fold change in expression. NKX3.1 ChIP: extracts were analyzed by ChIP assay using primers against NKX3.1-ARE site. Constitutive recruitment of AR to the ARE was dependent on endogenous p52 expression in C4-2 cells. D, immunoblotting of above cell lysates with p52 and AR antibodies.

Discussion

Prostate cancer is the most common type of cancer in American men, and failure of androgen deprivation therapy leads to castration-resistant disease progression. Accumulating evidence suggests that ligand-independent activation of AR and development of apoptosis-resistant cells contribute to CRPC. AR activation in CRPC may occur by a variety of mechanisms that alter the sensitivity or specificity of AR. Previous studies showed that overexpression of NF- κ B2/p52 induced castration-resistant growth of androgen-sensitive LNCaP cells *in vivo*. In this study, we investigated the potential role of p52 in activation of AR and show that p52 activates AR by interacting with its NH₂-terminal domain. p52 not only recapitulates DHT-mediated AR activation but also has a unique action in that p52-activated AR is preferentially recruited to the distal enhancer of the PSA promoter and is assembled into transcriptional complexes with coactivators such as p300 to optimize target gene transcription under androgen deprivation. These findings were confirmed in cell lines that possess Wt-AR (LAPC-4) as well as mutant AR (LNCaP). Downregulation of p52 in C4-2 cells, which constitutively express high levels of p52, led to abrogation of the constitutive activation of AR under conditions of androgen deprivation. These results suggest that NF- κ B2/p52 plays a major role in aberrant activation of the AR in prostate cancer cells after androgen ablation.

The classic pathway of NF- κ B is constitutively activated in prostate cancer, whereas the number of reports on activation of the alternative pathway is limited (31–33). We have reported that activated Stat3 may be involved in the processing of NF- κ B2/p100 in prostate cancer (32) and that overexpression of p52 induces androgen-independent growth of androgen-dependent LNCaP cells *in vivo* (33). We used several different approaches and provide direct evidence that p52 increases AR activation and transactivation of AR-responsive genes, may interact with the AR directly via its NTD, and enhances recruitment of coactivators such as p300 to the AREs of AR-responsive promoters. Previous studies have shown that p65 (RelA) induces expression and activation of the AR (42, 43), whereas our results show that p52 did not induce AR protein expression but induced nuclear translocation and enhanced transactivation of AR-responsive genes. The present study thus reveals a hitherto unknown positive link between the AR and the noncanonical NF- κ B pathway.

It is interesting to note that p52 recruits AR preferentially to the distal, but not to the proximal, promoter region of the

PSA gene, whereas DHT recruits AR to both distal and proximal enhancers of the PSA gene. These observations suggest that p52-activated AR may be conformationally different from DHT-activated AR and that it is likely to require other transcriptional mediators to “help” stabilize binding and potentiate transcription, as shown by results that p52 enhances recruitment of coactivators such as p300 to AR-responsive promoters and facilitates transactivation of androgen-responsive genes. Our finding is echoed by several recent studies (44, 45), which showed that the AR activated by aberrant signals such as neuropeptide and epidermal growth factor is recruited preferentially to AREI/II. It was shown that the modified AR efficiently translocates into the nucleus and assembles coactivator complexes different from those of DHT-bound AR, similar to our findings. These results are significant in showing that the AR activated by aberrant signals may have a conformation different from the DHT-bound one and could account for the different transcriptional outputs and hence phenotypes of hormone-sensitive versus CRPC.

In summary, we showed that NF- κ B/p52 activates the AR and enhances nuclear translocation and activation of AR by interacting with its NTD. p52 enhances the recruitment of coactivators such as p300 to the promoters of AR-dependent genes, resulting in increased transactivation of AR-responsive genes in androgen-deprived conditions. Our findings, together with previous reports that the levels of p52 are elevated in prostate cancer cells and that NF- κ B2/p52 promotes prostate cancer cell growth *in vitro* and *in vivo*, suggest that p52 may play a critical role in the progression of CRPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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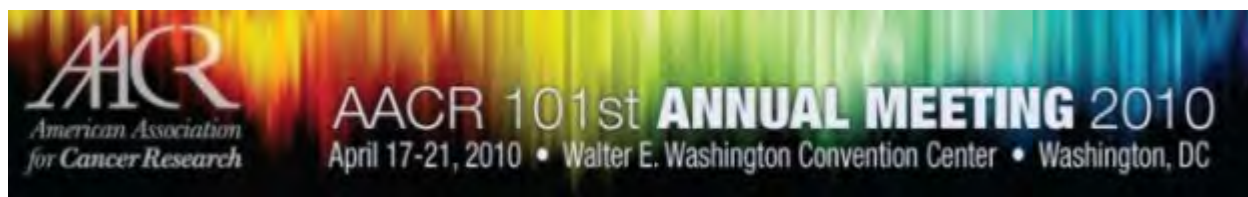
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Category: Endocrinology 1

Session Title: Receptor Signaling in Endocrine Related Cancers

#1709 Activation of androgen signaling axis by NF-kappaB2/p52 . Nagalakshmi Nadiminty, Wei Lou, Allen C. Gao. UC Davis Medical Center, Sacramento, CA .

INTRODUCTION: Castration-resistant progression of prostate cancer occurs in >80% of patients after failure of hormonal therapy but the underlying mechanisms are incompletely understood. Emerging evidence implies that ligand-independent activation of the androgen receptor (AR) is of critical importance during this process. Our previous studies show that the levels of NF- κ B2/p52 are elevated in prostate cancer cells and that active NF- κ B2/p52 promotes prostate cancer cell growth *in vitro* and *in vivo*. In this study, we examined the role of NF- κ B2/p52 in the aberrant activation of the AR during castration resistant progression, in contrast to the interaction between the AR and RelA/p65.

METHODS: The effects of NF- κ B2/p52 on cell survival, growth and androgen responsiveness were examined in prostate cancer cells. Modulation of LNCaP tumor growth by p52 was examined in intact and castrated male nude mice. Effect of p52 or p65 on AR activation was examined using luciferase assays, EMSA, ChIP assays and ELISA.

RESULTS: Expression of NF- κ B2/p52 enhanced androgen-sensitive LNCaP human CaP cell growth and clonogenic ability in androgen-deprived conditions *in vitro*. LNCaP cells expressing p52 exhibited protection from apoptotic cell death and cell cycle arrest induced by androgen-deprivation. Adenoviral mediated p52 expression in LNCaP cells induced tumor growth in castrated male nude mice. NF- κ B/p52 induced the activation of the AR resulting in increased transactivation of AR-responsive genes such as PSA and NKX3.1 in a ligand-independent manner. NF- κ B2/p52 enhanced nuclear translocation and transactivation of AR by interacting with its N-terminal domain and enhanced the recruitment of co-activators like p300 to the promoters of AR-dependent genes. These results were confirmed in 3 different prostate cancer cell lines: LAPC-4 (wild type AR), LNCaP (mutant AR) and C4-2 (androgen-insensitive). Transfection of p52 into LAPC-4 and LNCaP cells (which express low levels of p52) showed increased activation of the endogenous AR. Downregulation of endogenous p52 in C4-2 cells resulted in abrogation of AR constitutive activation. In contrast, we observed that p65 exhibited transcriptional repression of the AR, and did not induce expression of AR-responsive genes, PSA and NKX3.1.

CONCLUSIONS: These findings demonstrate that overexpression of NF- κ B2/p52 induces castration-resistant growth and aberrant activation of the AR in LNCaP cells. NF- κ B2/p52 may promote castration resistant progression by activating the AR under conditions of androgen deprivation.

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Prostate Cancer: Basic Research VII

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ABERRANT ACTIVATION OF THE ANDROGEN RECEPTOR BY NF-KAPPAB2/P52

Nagalakshmi Nadiminty*, Wei Lou, Allen Gao, Sacramento, CA

INTRODUCTION AND OBJECTIVES: Castration-resistant progression of prostate cancer occurs in >80% of patients after failure of hormonal therapy but the underlying mechanisms are incompletely understood. Emerging evidence implies that ligand-independent activation of the androgen receptor (AR) is of critical importance during this process. Our previous studies show that the levels of NF- κ B2/p52 are elevated in prostate cancer cells and that active NF- κ B2/p52 promotes prostate cancer cell growth in vitro and in vivo. In this study, we examined the role of NF- κ B2/p52 in the aberrant activation of the AR during castration resistant progression, in contrast to the interaction between the AR and RelA/p65.

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CONCLUSIONS: These findings demonstrate that overexpression of NF- κ B2/p52 induces castration-resistant growth and aberrant activation of the AR in LNCaP cells. NF- κ B2/p52 may promote castration resistant progression by activating the AR under conditions of androgen deprivation.

Source of Funding: NIH CA118887 and CA109441

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HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN H1 CONFERS TRANSCRIPTION AND TRANSACTIVATION OF ANDROGEN RECEPTOR: IMPLICATIONS FOR DISEASE PROGRESSION IN AFRICAN AMERICAN MEN

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INTRODUCTION AND OBJECTIVES: African American men (AA) have twice the incidence and mortality of prostate cancer (CaP)

than Caucasian American (CA) men and men of other ethnic minority groups. The causes of this ethnic disparity in clinical manifestation and outcome of the disease are not well understood.

METHODS: We identified selective expression of heterogeneous nuclear ribonucleoprotein H1 (hnRNPH1) in prostate tumor cells of AA men in comparison to CA men by employing a combined approach of laser capture microdissection (LCM), suppressive subtractive hybridization (SSH), and custom race-based CaP cDNA microarray on fresh specimens. Quantitative RT-PCR and ethnicity-based tissue microarray (TMA) analyses revealed selective nuclear accumulation of hnRNPH1 in tumor cells compared to adjacent normal epithelium and benign prostatic hyperplasia (BPH).

RESULTS: In addition to selective expression, hnRNPH1 up-regulates transcription, physically interacts with, and confers hormone-dependent (HD) and independent (HI) transactivation of androgen receptor (AR) in CaP cells. Further, our reporter, ChIP, and EMSA analyses demonstrate that hnRNPH1 binds to androgen response elements (AREs) on promoter and enhancer elements of PSA gene and the ligand binding domain-encoding exons D, E, and H of the AR gene, suggesting it acts as a coactivator of AR in CaP cells. Interestingly, siRNA silencing of hnRNPH1 caused growth arrest and enhanced cytotoxicity of Bicalutamide in AR-expressing PC cells.

CONCLUSIONS: Our findings support a model in which hnRNP H1 is an exclusive auxiliary factor for AR to elicit androgen-specific transcriptional regulation of androgen-regulated genes and development of drug resistance. Given heterogeneity of CaP and that AR is implicated in androgen independent progression of CaP, the results demonstrate a previously uncharacterized mechanism for AR-hnRNPH1 axis in disease progression and hormone refractory via enhancing HD and HI mediated transcription and transactivation of AR in a subset of prostate tumor cells in AA men. The results not only reveal racial differences in the biology of PC, but also provide, for the first time, a new frontier for the development of diagnostic, preventive, and/or targeted therapeutic strategies to circumvent disease progression in AA men.

Source of Funding: None

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EVIDENCE FOR A CENTRAL ROLE FOR PELP-1 IN ANDROGEN RECEPTOR MEDIATED GENOMIC SIGNALING IN PROSTATE CANCER.

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INTRODUCTION AND OBJECTIVES: Androgen receptor (AR) signaling is essential for prostate cancer development, growth, and progression at all stages of disease. AR signaling occurs via both genomic and non-genomic pathways and is mediated by AR interaction with cofactors. One such factor, a scaffolding protein, PELP-1 (Proline, Glutamic acid, Leucine rich Protein 1), has been shown to interact with AR and may play a role in AR-mediated non-genomic signaling. Since PELP-1 has been also shown to be responsive to estrogen receptor beta, we examined whether PELP-1 is involved in AR-mediated genomic signaling mediated by estradiol (E2) and by androgens.

METHODS: Prostate cancer cell lines expressing AR were treated with various concentrations of E2 (0.1-100nM) or androgen (DHT/R1881 (0.01-10nM)) and evaluated for proliferation with MTT assays. Immunofluorescent studies were performed to examine endogenous AR translocation to the DAPI-stained nucleus. Co-immunoprecipitation assays were performed with each combination of immunoprecipitating antibody and immunoblotting antibody. siRNA pools were obtained from Dharmacon and have been shown to knock down more than 90% of the targeted expression.

RESULTS: Upon addition of androgens or E2, AR translocates to the nucleus and activates transcription of androgen-responsive